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Biphasic plasticity of dendritic fields in layer V motor neurons in response to motor learning

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Abstract

Motor learning is associated with plastic reorganization of neural networks in primary motor cortex (M1) that advances through stages. Dendritic spines grow initially followed by pruning and maturation approximately one week after training ended. A similar biphasic course was described for the size of the forelimb representation in M1. This study investigates the evolution of the dendritic architecture in response to motor skill training using Golgy-Cox silver impregnation in rat M1. After learning of a unilateral forelimb-reaching task to plateau performance, an increase in dendritic length of layer V pyramidal neurons (i.e. motor neurons) was observed that peaked one month after training ended. This increment in dendritic length reflected an expansion of the distal dendritic compartment. After one month dendritic arborization shrinks even though animals retain task performance. This pattern of evolution was observed for apical and basal dendrites alike - although the increase in dendritic length occurs faster in basal than in apical dendrites. Dendritic plasticity in response to motor training follows a biphasic course with initial expansion and subsequent shrinkage. This evolution takes fourth as long as the biphasic reorganization of spines or motor representations.

Introduction

The primary motor cortex (M1) is involved in learning novel movement sequences, possibly as a site where the motor memory trace is formed [1,2]. In rodents that learn a motor task [3], plastic changes within M1 have been observed in the form of structural modification in dendrites [4] and their spines [5], in gene expression [6,7], synaptic weights [8] and motor maps [9]. With respect to the temporal profile of plastic changes, an initial phase of growth is followed by a subsequent phase of maturation [10]. An initial increase in spine formation for example is followed by an enhanced turnover that reduces the number of spines to baseline levels [5]. Whereas this concept of biphasic reorganization has been well demonstrated for spines [5], synaptic weights [11] and motor maps [12] it is unknown whether learning-induced growth of dendritic fields [4,13,14] is a lasting phenomenon or if a pruning occurs after a delay. To address this question, we measured dendritic morphology of layer V motoneurons at different time-points (day 0, day 30 and day 60) following acquisition of a skilled reaching task in rats. The somato-dendritic compartment was visualized using a Golgi-Cox silver impregnation and neurons were three-dimensionally reconstructed using a Neurolucida system.

Materials and Methods

1. Animals and experiments

Naïve adult 10-12 weeks old male Long-Evan rats ($n = 24$; 220 - 270 g; Centre d'Elevage R. Janvier, Le Genest - St. Isle, France) were used for this study. Animals were housed in cages in groups of three individuals in a 12/12-hour light/dark cycle (light on: 8 pm, off: 8 am). Training sessions were performed at the beginning of the dark phase. Animals were food-deprived for 24 hours prior to the first training session. Daily food supplements (ca. 50 g/kg of standard diet) were given after the reach training session to maintain constant body weight. Access to water was ad libitum. All experiments were conducted in accordance with Swiss regulations and were approved by the Committee for Animal Experimentation of the Canton of Zürich.

2. Experimental setup and behavioral experiments

Behavioral tasks were performed as previously described [12]. The training cage was a 15 x 40 cm chamber (height 30 cm) with a vertical window (1 cm wide, 5 cm high, lower edge 2 cm above ground) in the front wall and a small light sensor in the rear wall (7 cm above ground).

Two different behavioral conditions were compared: a motor skill learning paradigm (skilled reaching task; SRT) and controls with the operant but without the motor elements (control group; CG). These rats were exposed to the same training cage and had accessed a food pellet by tongue (pre-training). During pre-training animals learned to open the motorized sliding door that covered the front window by nose-poking the sensor in the rear. Opening the window gave access to one food pellet (45 mg, Bio-serve, Frenchtown, NJ, USA) located on a small horizontal board in a

distance of 0.5 cm relative to the outside edge of the window. During pre-training, pellets were retrieved by tongue. Upon retrieval, a pellet dispenser automatically replaced the pellet. For SRT animals, the first training session was done after five days of pre-training. Control animals (n=6) were killed after the fifth session of pre-training. For SRT and controls, forelimb preference was determined by placing the food pellet in a distance of 10 mm in front of the window. In this position pellets were only retrievable by using the forelimb. Animals were allowed to perform 20 reaching attempts - the paw that was used more frequently than the other one was defined as the preferred side.

In SRT animals (n=6) pre-training was followed by motor training that was initiated by removing the board and placing the pellet on a small vertical post 1.5 cm away from the window. The pedestal was shifted to one side of the window to allow for reaching with the preferred limb only. Because the diameter of the post was approximately that of the pellet, the pellet was in an unstable position easily kicked off the post. To retrieve the pellet rats had to extend the forelimb towards the target, pronate, open the paw, grasp, and pull the forelimb back while supinating to bring the pellet towards the mouth [3]. Each reaching trial was scored as “successful” (reach, grasp and retrieve) or “unsuccessful” (pellet pushed off pedestal or dropped during retraction). Each session consisted of 100 door openings (= trials). The improvement of reaching performance between sessions was defined as the success rate, i.e. number of successful trials/100 trials. Altogether eight training sessions were performed within consecutive days. Animals of the SRT+0 group (n=6) were killed within 15 minutes after the end of the eighth training session. For the SRT+30 (n=6) and the SRT+60 group (n=6), animals remained in their home cages without training for 30 and 60 days, respectively. After the home cage period, animals performed 100 reaching trials to measure reaching performance and were killed within 15 minutes after the

end of training. Because the time lag between reaching and euthanization was short (≤ 15 min from the start of the session) influences of task performance on dendritic morphology are unlikely: structural plastic changes in dendrites are known to occur only after several hours to days [15].

3. Histology and Morphological analysis

Animals were then deeply anesthetized (pentobarbital; 50 mg/kg i.p.; Kantonsapotheke Zurich, Switzerland) and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA). For analysis, only the hemisphere contralateral to the trained paw (for SRT) or contralateral to the preferred paw (for controls) was taken into account. To identify the hemisphere of interest after processing, hemisphere ipsilateral to trained/preferred paw was marked with a horizontal cut. Furthermore, a vertical cut was performed to mark the position of bregma. The brains were immersed whole in 20 ml of Golgi-Cox solution (FD Rapid GolgiStain™ Kit, FD NeuroTechnologies Inc., Columbia MD, USA). The brains were left in the solution for 14 days before being placed in a 30% sucrose solution for 2–5 days. Coronal sections (180 μ m) were prepared using a vibratome (Microm HM 650 V, Thermo Scientific, Walldorf, Germany). For each animal, brain sections containing the forelimb representation of M1 (3 mm ant. to 0.5 mm anterior to bregma) were collected. Sections were mounted with Permount mounting medium (bioWorld, Dublin, Ohio, USA) and analyzed using a microscope (Axioplan II, Zeiss AG, Jena, Germany; equipped with a motorized x-y stage; 40x/0.5 EC Plan-Neofluar objective). M1 was identified with respect to its characteristic cyto-architecture [16]. Layer V pyramidal neurons were traced using the Neurolucida software (Version 8.21.6, MicroBrightField Inc., Williston, VT, USA). Only neurons fulfilling the following criteria were sampled: the cell had to be well impregnated and not obscured by

blood vessels, astrocytes, or heavy clusters of dendrites from other cells and the apical and basilar arbors had to appear to be largely intact and visible in the plane of section. For every animal, 10 neurons per hemisphere were reconstructed. Neurons were sampled equally along the rostro-caudal axis of M1 (i.e. one neuron within each section). The researcher performing the reconstructions (C. Gloor) was blinded with respect of group identities.

4. Statistical analysis

Tracings were analyzed using NeuroExplorer version 4.7 (MicroBrightField Inc., Williston, VT, USA), statistical analyses were performed using Prism version 5.0 (GraphPad Inc., San Diego, CA, USA) and SPSS Statistics 22.0 (IBM Corp., Armonk, NY, United States). Learning curves were compared using 2-way repeated measures ANOVA with *group* (SRT, SRT+30 and SRT+60) as between- and *session* (training day 1-8) as within-subject factor. Morphological data were analysed using 1-way ANOVA, animals (i.e. the average of 10 cells per rat) have been used as subjects for the analysis. For the parameter “dendritic length”, the dendritic compartment (apical vs. basal) was included as independent variable. For the parameter “number of branches/branch order”, ANOVAs were corrected for multiple comparisons. Post hoc tests were performed using Bonferroni correction for multiple comparisons. Numerical results are expressed as mean and standard error of the mean (SEM).

Results

All animals learned to reach (**Figure 1**) without differences between groups ($F(1,2) = 1.3$; $p = 0.3$), there was no significant interaction of group \times time ($F(8,1) = 1.7$, $p = 0.1$). In the SRT+30 and SRT+60 group, the level of performance was maintained even though training was stopped on day 8.

Total dendritic length (TDL) of layer V motor neurons in the trained hemisphere significantly differed between groups ($F(3,3) = 17.6$; $p < 0.0001$; **Figure 2A**). Bonferroni-corrected post hoc tests confirmed that TDL was smallest in the CG group ($3616\mu\text{m} \pm 158\mu\text{m}$; $p < 0.001$ for CG vs. SRT, vs. SRT+30 and SRT+60) and largest in the SRT+30 group ($5104\mu\text{m} \pm 147\mu\text{m}$; $p < 0.001$ for SRT+30 vs. CG, vs. SRT and SRT+60). No significant difference was found between the SRT and SRT+60 group; both showed an intermediate TDL (SRT: $4337\mu\text{m} \pm 148\mu\text{m}$; SRT+60: $4481\mu\text{m} \pm 123\mu\text{m}$). Larger TDL was caused by increased arborization of distal dendrites as the cumulative number of branches/branch order significantly differed between groups beyond the fifth generation of branches (ANOVA corrected for multiple comparisons; generation 4: $p = 0.2$; generation 5: $p = 0.096$; **Figure 2B**). Exemplary two-dimensional projections of reconstructed motor neurons are shown in **Figure 2C**.

The ANOVA including the *dendritic compartment* (apical vs. basal), *group* and their interaction as independent variables showed no significance for the interaction, which was therefore dropped. *Compartment* by itself was significant ($F(1,1)=214.1$, $p < 0.0001$) and group showed a trend towards significance ($F(2,2)=3.10$, $p < 0.0598$). We then analyzed each compartment separately: For the apical dendrite, dendritic length (DL) was significantly different between groups ($F(3,3) = 15.1$; $p < 0.0001$;

Figure 3A). Bonferroni-corrected post hoc tests were performed for every possible combination of groups. DL is largest for the SRT+30 group ($p < 0.001$ for SRT+30 vs. CG, vs. SRT and vs. SRT+60). DL in the SRT+60 group is larger than CG ($p < 0.001$), no difference exists between SRT and CG. Significant differences in cumulative number of branches/branch order are present starting from the fifth generation of branches (ANOVA corrected for multiple comparisons; generation 4: $p = 0.11$; generation 5: $p = 0.038$; generation 6: $p = 0.006$; **Figure 3A**). For basal dendrites, the difference between *groups* was also significant ($F(3,3) = 10.6$; $p < 0.0001$; **Figure 3B**). Bonferroni-corrected post hoc tests were performed for every possible combination of groups. Whereas, DL is smallest for CG ($p < 0.001$ for CG vs. SRT, vs. SRT+30 and vs. SRT + 60), only a non-significant trend can be observed for a peak in DL in the SRT+30 group. Significant differences in number of branches/branch order are present starting from the fifth generation of branches (ANOVA corrected for multiple comparisons; generation 3: $p = 0.68$; generation 4: $p = 0.09$; generation 5: $p = 0.02$; **Figure 3B**). In summary, whereas the largest increase in DL occurs within the first month after training in apical dendrites (i.e. between SRT and SRT+30), a substantial increase in arborization is already present after the training ended in basal dendrites (i.e. between CG and SRT).

Discussion

This study shows that increases in dendritic length of layer V motor neurons induced by learning a skilled forelimb task is transient. Overall dendritic length increases because novel branches are formed in the distal dendritic compartment. Branch formation peaks one month after completion of motor training. Dendritic arbors are subsequently pruned although motor task performance remains stable. This time course is observed for apical and basal dendrites alike - although the increase in dendritic length occurs faster in basal when compared to apical dendrites. Thus, structural changes in dendrites follow a biphasic course similar to the formation of spines or modifications in motor maps [10]. The time-scale of this course, however, is substantially longer (approximately four times).

The enlargement of dendritic fields in response to motor training reported here is in good agreement with the previous literature. Using the Golgy-Cox silver impregnation technique, an increased dendritic length and arborization in response to motor training has been described for apical [4] and basal dendrites [14] of motor neurons within layer V and II/III [17] contralateral to the trained limb. Silver impregnation randomly labels approximately one percent of cells within a tissue section [18]. Thus, this neuronal staining has no specificity for neurons that underwent plastic changes or were involved in the control of the trained limb. Recently, the results of earlier studies relying on Golgy-Cox staining were reproduced by Wang and colleagues [13], that used retrograde tracer injection into the cervical spinal cord to identify motor neurons that selectively control the forepaw. Training a reaching task induced an enlargement of dendritic length and number of branches only in these neurons but

not in motor neurons projecting to cervical segments controlling more distal muscles of the forelimb. Thus, despite its lack of staining specificity, the Golgy-Cox technique is well suited to display training-related morphological changes in motor cortical neurons.

For other phenomena interpreted as evidence of M1 plasticity, a biphasic time course of increase followed by decrease was described [10]. With respect to spine formation and the size of the forelimb representations in M1, a return to baseline was observed one week after the training ended [5,12]. Thus, in comparison with synapses and motor maps, the plastic modifications of dendritic morphology follow a slower time-course. Transient changes in response to motor training were also described for synaptic strength: the ability of M1 layer 2/3 neurons to undergo long-term potentiation (LTP, measured in a population of neurons) is reduced on day 5 (used up by learning) and then becomes restored at least two months after the onset of motor training [11]. The speed of this change is unknown because no measurements were conducted between day 5 and 2 months. Enlarged dendritic fields have been described two weeks after training onset [4,13,17] - a time-point at which spine density and map size already returned to baseline. The peak in dendritic arborization one month after training found here indicates a kinetic of plastic changes that is even slower than expected. Modifying dendritic architecture (formation of novel dendritic branches and increment in dendritic length of several hundred μm) may simply require more time and therefore lag behind. Alternatively, motor learning may consist of several processes that follow each other, e.g. reflecting initial acquisition and short-term consolidation in between sessions [2] followed by long-term consolidation of the sequence of movement elements [19,20]. Long-term consolidation may account for the preservation of skills for years without use of the skill. Dendritic

plasticity therefore may reflect a slow component of motor learning paralleling long-term consolidation [20,21].

Despite the different time-scales, the different phenomena of motor learning-associated modifications to structure and function of M1 circuits are correlated. Inducing LTP-like plasticity by stimulation of transcallosal fibers in rats increases forelimb representation size, dendritic length and the number of branches in layer V motor neurons [22]. Long-term depression has the opposite effect [23]. Interestingly, motor training induced enhancement of synaptic strength in horizontal connections of layer I in M1 of rats is accompanied by an increment in spine width one month after training onset [24]. Thus, in contrast to the formation of novel spines [5], changes in spine morphology follow a slow time course similar to the enlargement of dendritic fields. Whether a retraction of dendritic width occurs in parallel with dendritic pruning has to be clarified in further studies.

Reaching performance is maintained despite shrinking dendritic fields two months after training. Thus, it seems that dendritic arbors do not reflect the motor memory trace. Motor training induces an initial overshoot of structural dendritic plasticity followed by pruning or maturation of circuitry by selectively preserves functionally important elements. Whether this pruning is necessary for long-term consolidation of a skill remains to be shown.

Dendritic plasticity occurs in different compartments with different speeds: whereas a marked increase in basal dendrites occurs already during training, the growth of the apical dendrite follows a prolonged time course. Even though the physiological properties of basal and apical dendrites in layer V motor neurons are not well defined, this difference in plasticity suggests that both dendritic compartments play their particular functional role. Apical dendrites spread through different cortical

layers whereas basal dendrites are confined to layer V [25]. The complex architecture and higher degree of arborization of apical dendrites suggest a strong filtering of single synaptic events thereby allowing a broad integration of different inputs - whereas the simpler architecture of basal dendrites may allow an effective modulation of somatic excitability [26]. This view is in line with recent observations in layer V motoneurons of mice, showing a direct impact of synaptic inputs into basal dendrites on neuronal excitability, whereas the apical dendrite forms an independent compartment [27]. If these factors of input integration vs. output control are differentially modulated by motor learning has to be clarified in future work.

Methodological considerations

Layer V pyramidal neurons are generally difficult to study as the plane of cutting must be in the vertical plane of neurons to avoid truncation of distal terminal of apical dendrites. In our dataset apical tufts of motor neurons were at least partially truncated in some cells. However, we are confident that this limitation does not reduce the validity of this study. The learning-dependent enlargement of apical dendrites reported here is in good agreement with previous studies [4,13]. Furthermore, with respect to the average length of apical dendrites, neurons from our dataset seem to be well preserved when compared to earlier reports [4].

To differentiate plastic changes induced by learning from alterations induced by mere activation of a limb during a task, non-skill use paradigms are frequently used in motor learning studies. Such “activity controls” (e.g. rewarding reaching attempts by application of a food pellet directly into the animals mouth) are known to induce a certain enlargement of dendrites of layer V motor neurons. However, this enlargement is significantly smaller when compared to learning groups in both, apical [4] and basal dendrites [13,14]. Furthermore, this activity-induced enlargement of dendritic trees follows a different time-course with a pruning that is present after four weeks [28]. We therefore did not include a non-skill use practice group as control. Likewise, we did not include an age-matched control group as age-related changes in dendritic morphology develop over several years [29] – within the 1 to 2 months that are relevant for our study, no changes in dendritic morphology are expected [30,31].

Figures legends

Figure 1. Learning curves. All animals acquired the motor task. SRT+30 and SRT+60 groups retained the skill after 30 and 60 days of rest, respectively.

Figure 2. Motor learning-induced dendritic expansion of layer V neurons is reverted after two months. (A) Total dendritic length of layer V motor neurons in the trained hemisphere is enlarged in response to training (SRT). This enlargement peaks around one month (SRT+30) and subsequently reverses (SRT+60, $**p < 0.001$, SRT and SRT+60 are not significantly different). (B) The enlargement of dendritic fields is the consequence of increased arborization of distal dendrites. The number of cumulative branches/branch becomes significantly different starting from the fifth branching-generation. $**p < 0.001$. (C) Exemplary renderings of Neurolucida-reconstructed layer V motor neurons within the trained hemisphere (Scale bar 250 μ m).

Figure 3. Morphological plasticity modifications in dendritic sub-compartments. (A) In apical dendrites, dendritic length shows a peak after one month of training followed by a pruning at after two months. However, there is no increase immediately after the end of the training period (no difference between CG and SRT). The cumulative number of branches/branch is significantly different starting from the fifth branching-generation. $*p < 0.05$, $**p < 0.001$. (B) In basal dendrites, an increase of dendritic length occurs immediately after training ended. Only a non-significant trend is observed for increased dendritic length at 30 days (SRT+30). Dendritic length is smallest in controls when compared to the other groups. The cumulative number of branches/branch becomes significantly different starting from the fifth branching-generation. $*p < 0.05$.

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